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Assessing humoral and cell-mediated immune response in Hawaiian green turtles, *Chelonia mydas*

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Abstract

Seven immature green turtles, *Chelonia mydas*, captured from Kaneohe Bay on the island of Oahu were used to evaluate methods for assessing their immune response. Two turtles each were immunized intramuscularly with egg white lysozyme (EWL) in Freund's complete adjuvant, Gerbu, or ISA-70; a seventh turtle was immunized with saline only and served as a control. Humoral immune response was measured with an indirect enzyme linked immunosorbent assay (ELISA). Cell-mediated immune response was measured using in vitro cell proliferation assays (CPA) using whole blood or peripheral blood mononuclear cells (PBM) cultured with concanavalin A (ConA), phytohaemagglutinin (PHA), or soluble egg EWL antigen. All turtles, except for one immunized with Gerbu and the control, produced a detectable humoral immune response by 6 weeks which persisted for at least 14 weeks after a single immunization. All turtles produced an anamnestic humoral immune response after secondary immunization. Antigen specific cell-mediated immune response in PBM was seen in all turtles either after primary or secondary immunization, but it was not as consistent as humoral immune response; antigen specific cell-mediated immune response in whole blood was rarely seen. Mononuclear cells had significantly higher stimulation indices than whole blood regardless of adjuvant, however, results with whole blood had lower variability. Both Gerbu and ISA-70 appeared to potentiate the cell-mediated immune response when PBM or whole blood were cultured with PHA. This is the first time cell proliferation assays have been compared

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between whole blood and PBM for reptiles. This is also the first demonstration of antigen specific cell-mediated response in reptiles. Cell proliferation assays allowed us to evaluate the cell-mediated immune response of green turtles. However, CPA may be less reliable than ELISA for detecting antigen specific immune response. Either of the three adjuvants appears suitable to safely elicit a detectable immune response in green turtles.

Keywords: Green turtle; *Chelonia mydas*; Enzyme linked immunosorbent assay; Freund's adjuvant; Gerbu; ISA-70; Lymphocyte stimulation test

1. Introduction

Green turtles (*Chelonia mydas*) are endangered herbivorous marine reptiles that live in tropical and subtropical foraging pastures of algae and seagrasses throughout the world. A distinct population exists in Hawaii (Bowen et al., 1992) which nests mainly at French Frigate Shoals in the northwestern Hawaiian islands (Balazs, 1980).

Green turtles are adversely impacted by man-made factors (Balazs and Pooley, 1993) and diseases like fibropapillomatosis. Fibropapillomatosis (FP) causes debilitating external and internal tumors in green turtles worldwide (Herbst, 1994). In Hawaii, prevalence of FP is increasing or stable (Balazs, 1991). Fibropapillomatosis is probably caused by a virus (Herbst et al., 1995; Casey et al., 1997; Quackenbush et al., 1998) although the role of toxicants has also been explored (Aguirre et al., 1994).

Aguirre et al. (1995) suggested that turtles afflicted with FP were immunosuppressed. An ideal evaluation of the immune response of free-ranging green turtles would measure cell mediated and humoral immune response to a defined antigen. However, in order to make meaningful conclusions regarding the immune response of wild green turtles, assays to measure this response would first need validation in a captive setting.

There is considerable information on measuring humoral immunity of green turtles. Benedict and Pollard (1972) found that green turtles had three classes of immunoglobulins (5.7S, 7S and 17.6S) against two of which Herbst and Klein (1995) developed monoclonal antibodies. Both Benedict and Pollard (1972) using radioelectrocomplexing and Herbst and Klein (1995) using enzyme-linked immunosorbent assay (ELISA) showed that green turtles produce a detectable humoral immune response when exposed to a foreign antigen.

Information on the cell-mediated immune response of green turtles is more limited. McKinney and Bentley (1985) found that green turtle peripheral blood mononuclear (PBM) cells could be isolated and stimulated with mitogens in vitro although stimulation indices were low. Cell proliferation assays using PBM can produce variable results (Maluish and Strong, 1986). Hence, using CPA to compare cell-mediated immune response between groups of animals, such as turtles with and without FP, requires that stimulation indices be as high as possible in order to maximize the ability to detect significant differences. Cell proliferation assays using whole blood have been used in birds (Redig et al., 1984) and are simpler to perform than CPA using purified PBM. However, because the numbers of PBM used per animal are not standardized in whole blood preparations, comparisons between individuals and groups of animals using this method may be problematic.

The objectives of this study with captive green turtle were to: (1) assess whether a single exposure to an antigen would produce a detectable immune response using ELISA and CPA; (2) compare in vitro CPA using whole blood or PBM; and (3) determine which of three adjuvants could be safely used to provide an optimal antigen specific humoral and cell-mediated immune responses.

2. Materials and methods

2.1. Study animals

Seven immature turtles of undetermined sex were captured from Kaneohe Bay, Hawaii. Straight carapace length ranged from 40.8 to 54.1 cm (10–23 kg). Prior to being placed in captivity, turtles were carefully examined for absence of fibropapillomas on the skin, eyes, or glottis because Kaneohe Bay is an area endemic for FP (Balazs, 1991). Before immunization, turtles were allowed to adapt to captivity for at least 1–2 months in 9 m^3 tanks (2–3 turtles/tank) with circulating seawater and were fed a daily diet of squid and mackerel. Tank temperatures were recorded daily and ranged from 23 to 28°C (24.7±0.7). All procedures used in this study were reviewed and approved by the National Wildlife Health Center Institutional Animal Care and Use Committee and conformed to guidelines of the Animal Welfare Act.

Chicken egg white lysozyme (EWL) was chosen as the antigen because it is a complex, well characterized protein to which the turtles were not likely exposed. For adjuvants, Freund's complete adjuvant, Gerbu (CC Biotech, Poway, CA) and Montanide ISA-70 (Seppic, Fairfield, NJ) were selected. Freund's adjuvant (FCA) was used to provide a direct comparison with past studies involving experimental immunization of green turtles (Benedict and Pollard, 1972). Gerbu (Grubhofer, 1995) and ISA-70 (Yamanaka et al., 1992) were chosen for their propensity to elicit a less severe local inflammatory reaction at the injections site than FCA. Egg white lysozyme dissolved in phosphate buffered saline (PBS) was mixed with each adjuvant according to the manufacturers' instructions. The dose of Freund's adjuvant and ISA-70 varied because it was administered on a per/kg basis, and turtles gained weight through the study (Table 1).

In September 1997, six turtles (two for each adjuvant) were injected with 1 mg/kg of EWL in the ventral rear leg muscle mass; a seventh turtle was injected with PBS and served as a control (Table 1). A second immunization was given 21 weeks later at the same site. The dose of antigen was comparable to that used to elicit a detectable immune response to bovine serum albumin in green turtles after a single immunization (Benedict and Pollard, 1972). The study ended in March 1998.

Turtles were monitored daily for food consumption. Every 1–2 weeks, turtles were weighed with a spring scale, and 10 ml heparinized blood (18 USP heparin/ml) was collected from the cervical sinus using a sterile syringe and 0.9 mm×2.57 cm needle (Owen and Ruiz, 1980). During each procedure, the antigen injection site was examined visually and palpated for swelling or granuloma formation. Each blood sample was evaluated for hematocrit, estimated total solids, and a complete blood count (Work et al., 1998).

Table 1
Immunization protocol for green turtle immune response study[a]

Turtle	Primary immunization		Secondary immunization		Time of release	Comments
	Adjuvant	EWL	Adjuvant	EWL		
1	150 μl FCA	150 μl	220 μl FIA	220 μl	Week 25	FP in eye
2	100 μl FCA	100 μl	150 μl FIA	150 μl	Week 25	
3	2.5 ml Gerbu	0.5 ml	2.5 ml Gerbu	0.5 ml	Week 25	
4	2.5 ml Gerbu	0.5 ml	2.5 ml Gerbu	0.5 ml	Week 25	
5	480 μl ISA-70	120 μl	720 μl ISA-70	180 μl	Week 25	
6	800 μl ISA-70	200 μl	1.2 ml ISA-70	300 μl	Week 23	Weakness of limbs
7	3 ml PBS	NA	NA		Week 13	Weakness of limbs

[a]Primary immunization was on 15 September 1997, secondary was 21 weeks later. FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; PBS, phosphate buffered saline. Concentration of EWL for turtles 1, 2, 5, 6: 100 mg/ml; for turtles 3–4: 50 and 60 mg/ml for primary and secondary immunization, respectively.

2.2. Cell proliferation assays (CPA)

The cell-mediated immune response was evaluated using methods adapted from McKinney and Bentley (1985). Briefly, whole blood was diluted 1:2 in PBS, overlaid on an equal volume of ficoll 1077 (Sigma, St. Louis, MO), and centrifuged at $300\times g$ for 15 min. Peripheral blood mononuclear cells (PBM) were recovered from the ficoll–saline interface, washed three times in PBS, and resuspended to a final concentration of 4×10^6 cells/ml in RPMI-1640 supplemented with 2-mercaptoethanol (0.005 M) and sodium pyruvate (3 μg/ml). Cell viability was assessed with Trypan blue dye exclusion, and purity was determined by staining a film of the cell suspension with a Romanowsky-type stain (Fisher, Pittsburgh, PA), and classifying 200 cells under 1000× as mononuclear cells, granulocytes or RBC. Mean (±SD) viability of isolated PBM on the day of culture was 95±3% (range: 76–99%). The mean percentage of PBM on the day of culture was 96±0.03% (range: 82–100%). Cells were plated (100 μl/well) in 96-well microculture plates (Costar, Cambridge, MA).

Phytohaemagglutinin (PHA) and concanavalin A (ConA) (Sigma) were used as T-cell mitogens (Green and Cohen, 1979; Cuchens and Clem, 1979a; Benjamini and Leskowitz, 1994). Egg white lysozyme was used to assess antigen-specific response. Mitogens or EWL were mixed with RPMI containing 0.5% (w/v) of a powdered serum replacement (Albumax-I) (Gibco, Gaithersburg, MD) at concentrations of 1, 10 and 50 μg/ml for ConA, 0.1, 1, 10 and 100 μg/ml for PHA, and 0.01, 0.1, 1 and 10 μg/ml for EWL. Antigen and mitogens (100 μl/well) were dispensed in triplicate wells; triplicate wells containing cells only in RPMI supplemented with Albumax-I served as unstimulated controls for each assay.

Cell proliferation assays with whole blood were done using methods adapted from Redig et al. (1984). Briefly, whole blood was diluted 1:20 in RPMI and dispensed in triplicate at 100 μl/well. Mitogens were plated as above except that concentrations were 10, 50, 100 and 200 μg/ml for ConA and 1, 10 and 100 μg/ml for PHA. Triplicate wells containing diluted whole blood only in RPMI supplemented with Albumax-I served as unstimulated controls for each assay.

Plates were incubated for 90 h in a humidified atmosphere at 32°C and 8% CO_2. Cells were pulsed with 3.7×10^4 Bq of tritiated thymidine (185 GBq/mmol) (Amersham Life Sciences, Chicago, IL) in 50 μl RPMI/Albumax-I and harvested 18 h later onto glass filters. Filters were placed in scintillation fluid (Optifluor, Packard Instruments, Downers Grove, IL), and radioactive decay (disintegrations/min [DPM]) was quantified using a liquid scintillation counter. Mean DPM of triplicate wells for each mitogen or antigen concentration was divided by mean DPM of triplicate control wells to calculate the stimulation index (SI). Positive value for antigen specific stimulation in PBM cultures or whole blood were based on the EWL results for repeated experiments with the PBS control turtle. The positive value was calculated as the mean of peak SI plus two standard deviations of control turtle PBM cultured with EWL.

2.3. Preparation of rabbit anti-turtle IgY reagent

The globulin fraction from 12 ml of pooled plasma from immature green turtles from a fibropapilloma-free area was precipitated in half-saturated (50%) ammonium sulfate (Dessauer, 1970). The insoluble protein fraction was pelleted by centrifugation at $1500\times g$ for 10 min. The resulting precipitate was resuspended in 50% saturated ammonium sulfate and repelleted three additional times and then redissolved in PBS. The soluble globulin fraction was then desalted on a Sephadex G-25 column and concentrated in Amicon Microcon-100 tubes (Millipore, Bedford, MA). The total protein in the resulting sample was quantified using the BCA assay (Pierce, Rockford, IL).

A pooled globulin sample containing 250 μg of protein was diluted 1:2 in electrophoresis sample buffer (Bio-Rad, Hercules, CA) without mercaptoethanol and resolved on a 20 cm 5% acrylamide separator gel under non-reducing conditions using a discontinuous system (Laemmli, 1970). Appropriate known molecular weigh markers accompanied each run. Resolved protein bands were visualized by negative staining with copper. Two bands of ≈180 kDa, the size of full length Green Sea Turtle IgY (Herbst and Klein, 1995) were carefully excised, destained with Tris-glycine destain solution, minced, and recovered by electroelution.

The eluted protein specimens were dialyzed against PBS (pH 7.4), concentrated, diluted in reducing electrophoresis buffer, run on a 10% polyacrylamide minigel under reducing conditions with low and high molecular weight markers, and stained with Coomassie brilliant blue 250R. The reduced 180 kDa protein migrated as two bands representing the heavy and light chains, identifying the protein turtle IgY. Purified heavy chain was prepared from the isolated intact IgY by reduction electrophoresis, excision, electroelution and dialysis using the isolated heavy chain as a molecular weight marker. Electrophoretic analysis of the pooled IgY heavy chain preparation on a 10% acrylamide gel stained with Coomassie Brilliant Blue 250R demonstrated a single band at ≈68 kDa.

Polyclonal rabbit anti-turtle IgY antisera (RAT) was prepared at the Animal Resources Services at University of California, Davis. Briefly, a new Zealand White rabbit was prebled and then immunized three times with 150 μg of the 180 kDa IgY heavy chain emulsified in Freund's adjuvant at 2-week intervals. Each immunization was given as multiple (4–5) subcutaneous injections along the back. The initial dose was emulsified in an equivalent volume of Freund's complete adjuvant. Freund's incomplete adjuvant was

employed in all subsequent immunizations. The rabbit was anesthetized with a ketamine xylazine cocktail 2 weeks after the third immunization and exsanguinated by cardiocentesis. The anti-IgY heavy chain titer was determined by solid phase ELISA (Campbell et al., 1984).

2.4. ELISA

Humoral immune response of green turtles to EWL was measured with an indirect ELISA (Crowther, 1995). ELISA plates (Falcon, Becton Dickinson, Oxnard, CA) were coated with 50 μl/well of EWL (100 μg/ml) in PBS and air dried overnight at 27°C. After washing three times in (0.13 M NaCl) borate buffered saline (BB), wells were blocked for 1 h with 2% nonfat milk in high salt (0.5 M NaCl) borate buffer (HSBB). Plates were washed three times with HSBB and incubated for 1 h with 50 μl turtle plasma diluted 1:25 in 2% milk–HSBB. After three washes in HSBB, plates were incubated for 1 h with 50 μl/well of RAT diluted 1:400 in 2% milk–HSBB. Plates were washed three times with HSBB and incubated for 1 h with 50 μl/well goat anti-rabbit antibodies conjugated to horseradish perodixase (GHRP) (Alpha Diagnostics, San Antonio, TX) diluted 1:1000 in 2% milk–BB. After three more washes in BB, plates were incubated for 1 h with 100 μl/well 5-aminosalycilic acid (5-AS). Optical density (OD) was measured at 450 nm wavelength with an ELISA plate reader (Sigma). Reciprocal titers for selected samples represented the inverse of serial two-fold dilutions of plasma. A positive value for the ELISA value was set as the mean OD plus two standard deviations of pre-immunization plasma of seven turtles.

The following negative controls in duplicate EWL coated wells were used: (1) substrate; (2) substrate+GHRP; (3) substrate+5-AS+GHRP+RAT. Optical density readings were corrected for background by subtracting the mean OD of the six negative control wells.

2.5. Data analysis

Data were analyzed with a commercial software package (Sigmastat, SPSS, Chicago, IL). Multiple comparisons were done using repeated measures analysis of variance while *t*-test was used for pair-wise comparisons. Correlations were evaluated using Spearman correlation. Alpha for all comparisons was 0.05.

3. Results

3.1. Animals

No local inflammatory reaction was seen in all seven turtles after the primary immunization. After the secondary immunization, mild edema was palpated at the injection site which slowly regressed in the ensuing 2–3 weeks. Turtle 6 (EWL/ISA-70) developed progressive weakness of the forelimbs after the secondary immunization, and Turtle 7 (PBS control) developed progressive weakness of one front and one rear limb.

Both turtles became inappetent, anemic, and monocytotic. They were removed from the study and released after being given supportive care (Table 1). Hematology and clinical disposition of remaining turtles was unremarkable except for Turtle 1 which developed a 4 mm fibropapilloma in the medial canthus of the right eye. Throughout the study, turtles 1–6 gained an average of 5.8 kg; Turtle 7 lost 1.2 kg.

3.2. ELISA

The positive value for ELISA was OD=0.07. Antigen specific antibodies were first detected in all adjuvant groups at 6–9 weeks after the primary immunization (Fig. 1). Thereafter, both turtles immunized with EWL–FCA (Fig. 1A) or EWL–ISA-70 (Fig. 1E) maintained significant levels of circulating antibodies after primary immunization. In contrast, only one of two turtles receiving EWL–Gerbu developed detectable antibodies after the primary immunization (Fig. 1C).

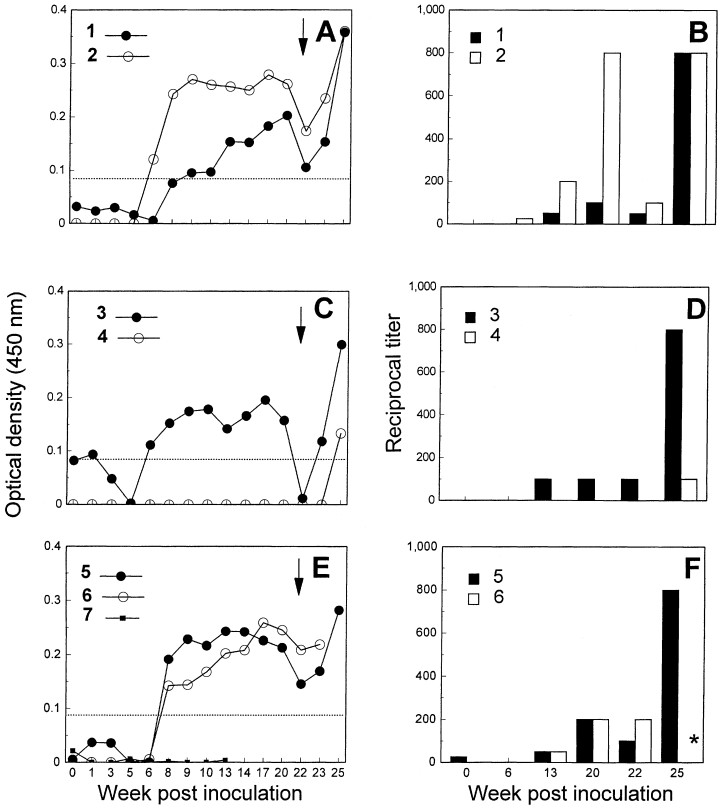

Fig. 1. ELISA absorbance readings (A, C, E) and reciprocal titers (B, D, F) for turtles given EWL with FCA (A–B); Gerbu (C–D) or ISA-70 (E–F). The dotted line in A, C and E represents the cutoff (OD=0.07) for antigen specific response. The asterisk in F represents a missing sample. The arrow indicates the secondary immunization.

Significant boosting of the antibody response was rapidly detected in all turtles given a second immunization regardless of adjuvant formulation. In addition, the turtle in the Gerbu adjuvant group which failed to show a primary response produced a low but significant antibody response following the secondary immunization (Fig. 1C). The highest titer (>1:800) observed after primary immunization was in a turtle receiving EWL–FCA (Fig. 1B). Most turtles had titers <1:200 after primary immunization. All except one turtle produced titers >1:800 after secondary immunization (Fig. 1).

3.3. Cell proliferation assay

High levels of proliferation were seen for PBM cultures of green turtles stimulated in vitro with ConA or PHA (Table 2). Optimal mitogen concentrations for PBM culture were 1–10 μg/ml for ConA and PHA after both the primary and secondary immunization

Table 2
Cell proliferation assay of mononuclear cells during the primary and secondary immune response of green turtles (pooled by adjuvant) immunized with three adjuvants

Mitogen[a]	Freunds			Gerbu			ISA-70			Control		
	Mean	SD	n[b]	Mean	SD	n	Mean	SD	n	Mean	SD	n
Primary immunization												
ConA 1	187.1	199.9	20	146.3	137.2	20	208.0	245.2	20	137.3	195.8	7
ConA 10	345.3	358.5	20	464.5	584.7	20	235.7	268.0	20	130.6	224.2	7
ConA 50	57.3	56.1	20	107.0	109.6	20	34.4	42.8	20	34.6	63.6	7
PHA 0.1	11.5	8.2	20	25.5	18.1	20	11.5	14.0	20	26.2	61.7	7
PHA 1	310.3	247.3	20	136.7	94.8	20	92.1	88.2	20	90.7	131.9	7
PHA 10	376.8	305.4	20	306.7	283.5	20	164.5	178.7	20	141.5	278.1	7
PHA 100	44.2	42.8	20	45.2	53.3	20	9.8	11.3	20	16.6	36.9	7
EWL 0.01	1.3	0.5	18	1.7	1.8	18	2.0	3.3	18	1.0	0.4	6
EWL 0.1	1.4	0.7	18	1.9	1.8	18	5.0	15.9	18	0.9	0.4	6
EWL 1	2.6	4.9	18	2.0	1.4	18	2.4	3.2	18	0.7	0.4	6
EWL 10	3.3	5.3	18	1.8	1.8	18	3.9	11.1	18	1.7	1.3	6
Secondary imunization												
ConA 1	197.8	174.2	6	134.2	73.7	4	78.5	96.5	4			
ConA 10	225.8	215.5	6	260.2	223.2	4	114.0	151.3	4			
ConA 50	41.9	49.3	6	51.0	39.6	4	17.9	26.0	4			
PHA 0.1	14.4	12.0	6	25.5	31.2	4	2.8	1.5	4			
PHA 1	224.3	147.5	6	150.2	60.2	4	26.0	23.3	4			
PHA 10	228.7	162.3	6	213.9	179.3	4	116.2	180.9	4			
PHA 100	44.9	39.6	6	40.2	36.3	4	4.9	5.6	4			
EWL 0.01	1.6	0.6	6	1.3	0.2	4	1.0	0.2	4			
EWL 0.1	1.6	0.5	6	1.3	0.4	4	1.3	0.6	4			
EWL 1	2.2	1.2	6	1.4	0.5	4	1.6	0.6	4			
EWL 10	4.2	3.6	6	3.4	3.6	4	3.2	3.3	4			

[a] Mitogens and concentration (μg/ml).
[b] Refers to number of triplicate assays.

Table 3
Cell proliferation assay of whole blood during the primary and secondary immune response of green turtles (pooled by adjuvant) immunized with three adjuvants

Mitogen[a]	Freunds			Gerbu			ISA-70			Control		
	Mean	SD	n[b]	Mean	SD	n	Mean	SD	n	Mean	SD	n
Primary immunization												
ConA 10	25.5	27.1	20	42.1	22.2	21	27.2	16.3	21	12.8	12.3	8
ConA 50	44.3	22.6	20	67.0	36.1	21	31.9	19.5	21	53.7	37.8	8
ConA 100	29.1	11.8	20	36.1	17.3	20	20.4	12.0	20	40.4	28.5	7
ConA 200	12.5	5.3	20	18.3	12.6	20	7.4	5.5	20	19.2	9.4	7
PHA 1	10.8	8.7	20	17.8	10.3	21	7.6	3.6	21	4.7	1.8	8
PHA 10	73.1	32.3	20	74.3	27.5	21	49.1	28.2	21	33.9	10.8	8
PHA 100	49.7	22.0	20	69.7	59.2	21	26.9	21.0	21	51.5	28.4	8
EWL 0.01	1.1	0.3	18	1.2	0.5	18	1.2	0.3	18	1.0	0.1	6
EWL 0.1	1.2	0.3	18	1.2	0.4	18	1.2	0.3	18	1.0	0.2	6
EWL 1	1.3	0.5	18	1.3	0.3	18	1.2	0.3	18	1.5	0.6	6
EWL 10	2.4	1.9	18	2.2	0.9	18	2.1	0.7	18	2.4	1.5	6
Secondary immunization												
ConA 10	42.6	22.5	6	31.3	10.0	6	21.7	12.8	5			
ConA 50	40.0	15.8	6	41.4	17.5	6	12.4	3.7	5			
ConA 100	20.4	8.9	6	21.7	15.5	6	4.9	1.3	5			
ConA 200	8.3	2.1	6	8.8	6.5	6	1.7	1.3	5			
PHA 1	16.7	3.6	6	13.3	7.3	6	8.5	2.7	5			
PHA 10	77.2	13.9	6	50.5	17.2	6	24.5	10.9	5			
PHA 100	39.0	13.9	6	37.9	26.8	6	10.6	4.1	5			
EWL 0.01	1.4	0.2	6	1.1	0.3	6	1.4	0.6	5			
EWL 0.1	1.5	0.4	6	1.2	0.2	6	1.3	0.4	5			
EWL 1	1.9	0.5	6	1.3	0.2	6	1.5	0.6	5			
EWL 10	3.8	2.2	6	3.1	2.0	6	1.9	0.9	5			

[a] Mitogens and concentration (μg/ml).
[b] Refers to number of triplicate assays.

with all adjuvant formulations. These concentrations were similar to optimal concentrations seen in the PBS control turtle (Table 2) and in healthy free-ranging green turtles (data not shown). Whole blood cultures had higher optimal mitogen concentrations ranging from 10 to 50 μg/ml for ConA and from 10 to 100 μg/ml for PHA (Table 3).

The positive value for antigen specific proliferation of PBM cultures was 4.3 (natural log=1.45) and 5.4 (natural log=1.6) for whole blood. Optimal EWL concentration for PBM ranged from 0.01 to 10 μg/ml; optimal EWL concentration for whole blood was 10 μg/ml except for one turtle (Tables 2–3).

For PBM, turtles 2 through 6 showed significant proliferation on at least one time point after primary immunization with EWL in FCA, Gerbu, or ISA-70 (Fig. 2). Turtle 2 (EWL/FCA; Fig. 2A) responded most consistently, with significant SIs in four of nine

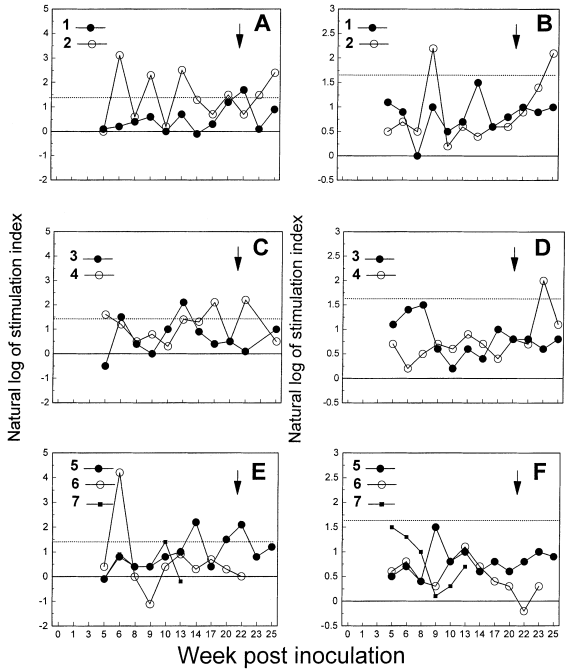

Fig. 2. Natural log of peak SI for PBM (A, C, E) or whole blood (B, D, F) cultured with EWL from turtles injected intramuscularly with EWL mixed with FCA (A–B), Gerbu (C–D), or ISA-70 (E–F). The dotted line represents the SI cutoff for PBM (SI=4.3; natural log=1.45) and for whole blood (SI=5.4; natural log=1.6). The arrow indicates the secondary immunization.

assays done during the primary response. The highest SI (SI=68) after primary immunization was seen in Turtle 6 (Fig. 2E) who received EWL in ISA-70. Turtle 1 (Fig. 1A) did not show a significant proliferative response after a single immunization. After the second immunization, significant proliferative responses were seen on at least one time period in both turtles receiving EWL in FCA. Significant proliferation was seen only in one turtle per group immunized twice with Gerbu or ISA-70 (Fig. 1C and E). For whole blood, significant EWL induced proliferation was seen only in Turtle 2 (FCA/EWL) after primary immunization and once each in turtles 2 and 4 (Gerbu/EWL) after secondary immunization (Fig. 2).

Significant differences in peak SI between adjuvants for PHA or ConA were seen only after primary immunization (Table 4). For PBM or whole blood cultured with PHA, peak SIs were significantly higher in turtles receiving FCA or Gerbu than for turtles receiving ISA-70 or the control. For whole blood cultured with ConA, peak SIs were significantly higher in turtles receiving Gerbu. There was no significant difference between adjuvant groups or controls for the antigen (EWL). When antigen (EWL) specific response was compared between primary and secondary immunization, the peak SI for EWL after secondary immunization were significantly greater in whole blood of turtles receiving

Table 4
Mean peak SI for whole blood and mononuclear cells after primary and secondary immunization of green turtles with various adjuvants and mitogens

Adjuvant[a]	Primary immunization				Secondary immunization			
	Mean[b]	SD	n[c]	CV	Mean	SD	n	CV
Mononuclear cells								
FCA/ConA	362.9	348.2	20	0.96	238.3	206.3	6	0.87
GER/ConA	475.3	580.5	20	1.22	293.4	181.9	4	0.62
ISA/ConA	250.3	273.3	20	1.09	114.4	150.9	4	1.32
CON/ConA	154.4	231.8	7	1.5				
FCA/PHA	408.6 a	311.7	20	0.76	252.4	143.0	6	0.57
GER/PHA	324.0 a	277.8	20	0.86	233.5	155.5	4	0.67
ISA/PHA	166.9	176.8	20	1.06	118.0	179.3	4	1.52
CON/PHA	149.0	275.1	7	1.85				
FCA/EWL	4.1	5.5	18	1.35	4.4	3.5	6	0.80
GER/EWL	3.1	2.2	18	0.72	3.5	3.5	4	0.99
ISA/EWL	5.9	15.8	18	2.67	3.6	3.0	4	0.82
CON/EWL	1.9	1.2	6	0.64				
Whole blood								
FCA/ConA	48.6	25.1	20	0.52	47.5	19.6	6	0.41
GER/ConA	62.9 a	20.6	20	0.33	42.6	16.8	6	0.39
ISA/ConA	36.3	18.0	20	0.50	22.2	12.1	5	0.55
CON/ConA	50.2	38.1	7	0.76				
FCA/PHA	76.2 a	31.0	20	0.41	77.2	13.9	6	0.18
GER/PHA	80.3 a	35.3	20	0.44	53.7	22.3	6	0.42
ISA/PHA	51.2	27.2	20	0.53	24.5	10.9	5	0.44
CON/PHA	53.6	30.1	7	0.56				
FCA/EWL	2.5	1.9	18	0.77	3.8	2.2	6	0.57
GER/EWL	2.2	0.9	18	0.41	3.1	2.0	6	0.67
ISA/EWL	2.1	0.7	18	0.34	1.9	0.8	5	0.43
CON/EWL	2.6	1.4	6	0.53				

[a] FCA, Freunds; GER, Gerbu; ISA, ISA-70; CON, Control; CV, coefficient of variation.
[b] Numbers within a column of a mitogen block are significantly different ($p<0.05$).
[c] Refers to numbers of triplicate assays.

FCA. Peak SI for EWL for remaining adjuvant groups for PBM or whole blood did not differ significantly between primary and secondary immunization. Overall, CPA results with PBM had higher coefficients of variation than whole blood. In contrast, SIs for whole blood were generally lower than PBM.

For PBM, SI for ConA and PHA were significantly correlated in all turtles; significant correlations were seen in whole blood only for turtles 1, 3, 4, 6, and 7 (Table 5). There was no significant correlation between mitogen and antigen specific response for PBM. For whole blood, significant correlations were seen between responses with ConA and EWL for turtles 3, 5 and 6 and between responses with PHA and EWL for Turtle 6. No significant correlation was seen between cellular response to EWL and humoral immune response in either whole blood or mononuclear cells (Table 5).

Table 5
Correlation coefficients for mononuclear cells or whole blood and antigen specific or humoral immune response

Turtle	ConA vs. PHA	EWL vs. ELISA	ConA vs. EWL	PHA vs. EWL
Mononuclear cells				
1	0.75*	0.36	0.10	0.14
2	0.61*	0.18	0.44	0.26
3	0.99*	0.18	0.08	0.08
4	0.69*	−0.40	−0.10	−0.36
5	0.97*	0.08	0.50	0.50
6	0.97*	0.36	0.58	0.36
7	1.00*	NA	NA	NA
Whole blood				
1	0.88*	−0.02	0.26	0.17
2	0.35	0.19	0.20	0.36
3	0.82*	−0.41	0.79*	0.39
4	0.71*	0.39	−0.21	0.20
5	0.33	0.46	0.82*	0.08
6	0.90*	−0.52	0.76*	0.75*
7	0.89*	NA	NA	NA

* Correlations are significant ($p<0.05$).

4. Discussion

This study confirmed that green turtles can mount an antigen-specific humoral immune response after one injection of antigen. Benedict and Pollard (1972) also detected antibody in green turtles after a single immunization. However, this is the first demonstration of an antigen specific cell-mediated immune response induced by a single immunization in reptiles. The kinetics of the antigen specific response in our study (∼6 weeks) were comparable to those reported by Benedict and Pollard (1972) but slower than that seen by Herbst and Klein (1995) (3–5 weeks) who immunized their turtles biweekly and detected immune response with monoclonal antibodies. The duration of the primary response was comparable to that reported by Benedict and Pollard (1972) although our turtles were monitored for a shorter time span. As a group, reptiles respond to antigenic stimulus more slowly than birds or mammals (Jurd, 1994). Factors that influence the immune kinetics of reptiles include type of antigen, use of adjuvants and temperature (Ambrosius, 1976).

Antibody titers in this study were rarely >1:200 after primary immunization. In some turtles, the titers increased to >1:800 after the secondary immunization. Herbst and Klein (1995) detected titers of up to 1:800 in turtles repeatedly immunized with DNP–BSA while Benedict and Pollard (1972) using radioelectrocomplexing detected titers >1:400 in some turtles immunized once with DNP–BSA. Like Benedict and Pollard (1972), we demonstrated that green turtles can mount an anamnestic response. The transitory drop in antibody levels after boosting was not encountered by Benedict and Pollard (1972). The synchrony and timing of this drop may have been due to clearance of immune complexes from circulation following the secondary immunization. We did not monitor IgM (17.6S)

specific response in green turtles because reagents for this assay were unavailable. Herbst and Klein (1995), using ELISA, had difficulty measuring IgM response in green turtles.

An antigen specific cell-mediated immune response in PBM was detected in green turtles immunized with all three adjuvant formulations, albeit intermittently. In comparison, antigen specific cell-mediated immune response for whole blood was almost undetectable. The lack of correlation between antigen-induced cell-mediated immune response and antibody response or mitogen induced response indicates that both parameters should be measured to obtain an assessment of overall immune responsiveness in the green turtle. Although the antigen specific SIs were significant, the low level of stimulation and the intermittent nature of this response indicates that this assay may not be sufficiently robust to reliably detect differences between normal and partially immunocompromised turtles.

In contrast to the low antigen-specific proliferative response, the mitogenic response of green turtles observed here was quite vigorous. This level of responsiveness supports the application of mitogen stimulated cell proliferation assays to assess the overall cell-mediated immune response in green turtles. Peak SIs for green turtle PBM cultured with ConA or PHA were higher than those for PBM from Florida green turtles (McKinney and Bentley, 1985) or from alligator (Cuchens and Clem, 1979a), thymocytes from the lizard *Chalcides ocellatus* (El Deeb and Saad, 1987); or splenocytes from the snake, *Psammophis sibilans* (Farag and El Ridi, 1986). Differences in cell types and technique probably account for the observed variations.

Albumax-I has been used successfully as a substitute for serum supplementation in cell culture (Grande et al., 1997). In addition to providing high stimulation indices, Albumax-I offered the advantage of eliminating batch to batch variation in fetal bovine serum that often complicates cell proliferation assays. The 90 h time period chosen for pulsing gave optimal stimulation indices and was based on earlier pilot trials where pulsing was done from 24–120 h.

The consistent correlation between response to PHA and ConA for green turtle PBM suggests we were working with a single subpopulation of cells analogous to PHA-inducible T-like lymphocytes of alligators (Cuchens and Clem, 1979b) or thymus dependent ConA- or PHA-inducible cells in frogs (Green and Cohen, 1979). Evaluating B-cell proliferation would have necessitated immunizing turtles with a thymus independent antigen and assessing cell-mediated response using B-cell specific mitogens such as lipopolysaccharide (Benjamini and Leskowitz, 1994). However, we were primarily interested in evaluating T-like cell response because our eventual goal is to use these assays to assess immunology of green turtle FP, and T-cells play a major role in tumor immunology (Greenberg, 1994).

Nevertheless, clarification of the status of lymphocyte subpopulations of green turtles is clearly needed. Investigations using glasswool (Cuchens and Clem, 1979b), nylon wool (Manickasundari et al., 1984), anti-IgY antibodies (Negm and Mansour, 1982), anti-thymocyte antibodies (Mansour et al., 1980; Negm and Mansour, 1983; El Deeb et al., 1986; El Ridi et al., 1987), and mitogens (El Deeb and Saad, 1987) provide compelling evidence of T- and B-like cells in reptiles and amphibians. Similar methods could be used to fractionate green turtle lymphocytes in order to define populations of ConA and PHA responsive cells.

This study is the first comparison of cell proliferation using whole blood or mononuclear cells in reptiles. Stimulation indices for green turtle PBM were much higher than for whole blood. While results from CPA in whole blood had lower variability, whole blood was unsuitable for detecting antigen-specific cell-mediated immune response. The lack of correlation between peak SI for ConA and PHA in whole blood suggested that mixed blood cells may provide a less reliable measurement of the proliferative response of an individual.

One turtle developed FP while in captivity. This turtle developed a detectable primary antibody response and displayed a typical booster response after secondary immunization. A significant antigen-specific proliferation response was detected after the secondary immunization, and a high mitogen response was seen in this turtle for both ConA and PHA during both the primary and secondary response. These observations suggest that if immunosuppression occurs in turtles with FP, it is likely a sequela to the disease rather than a predisposing factor.

The absence of significant adjuvant associated reaction at the injection site along with weight gain of all but the control turtle suggests that any of the three adjuvants (FCA, Gerbu, ISA-70) could be used safely in green turtles. The control turtle and another injected with ISA-70 developed similar clinical signs of physical impairment suggesting that factors other than the adjuvant or antigens were responsible for their debilitation. The control turtle was re-captured in Kaneohe Bay 2 months after release, had gained 4 kg, and had full function of all limbs. The second turtle has not been recaptured.

Freund's complete adjuvant and ISA-70 consistently induced comparable levels of antibody response in green turtles after a primary and secondary immunization. Thus, for monitoring humoral immunity in green turtles after a single immunization, FCA or ISA-70 may be preferable to Gerbu. All three adjuvants were comparable in terms of priming of antigen-specific cell proliferation responses in PBM. Gerbu and FCA appear to potentiate the proliferative response of PBM cultured with PHA. In the case of FCA, this is probably due to lymphocyte expansion by the adjuvant (Hibbs et al., 1972), and should be considered when cell-mediated immunity is assessed using PHA stimulation in green turtles immunized with these adjuvants. However, all three adjuvants appear suitable for eliciting antigen specific cell-mediated immune response in green turtles.

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